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Association between β-Lactamase-Encoding bla_{OXA-51} Variants and DiversiLab Rep-PCR-Based Typing of *Acinetobacter baumannii* Isolates

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This study investigated the correlation between bla_{OXA-51} variants and *Acinetobacter baumannii* worldwide clonal lineages 1 to 8 (WW1 to -8). The $bla_{OXA-51-like}$ genes of 102 *A. baumannii* isolates were sequenced. Using DiversiLab repetitive-sequence-based PCR (rep-PCR) typing, 92 of these isolates had previously been assigned to WW1 to -8 and 10 were unclustered. Clustering of DNA sequences was performed using the neighbor-joining method and the Jukes-Cantor phylogenetic correction. bla_{OXA-51} variants were in good correlation with DiversiLab-defined clonal lineages. Sequence-based typing of bla_{OXA-51} variants has the potential to be applied for epidemiologic characterization of *A. baumannii* and to identify worldwide clonal lineages 1 to 8.

Acinetobacter baumannii is a Gram-negative hospital-acquired pathogen which commonly causes pneumonia, bloodstream infections, meningitis, wound infections, and urinary tract infections, especially in patients with impaired host defenses (3, 14). Until recently, the majority of *A. baumannii* isolates, while being resistant to many antimicrobial classes (fluoroquinolones, tetracyclines, cephalosporins, and aminoglycosides), remained susceptible to carbapenems (3). However, today carbapenem resistance is more frequently encountered, with rates of up to 70% of isolates reported in some countries (3, 11, 13, 14, 19). Predominantly in *A. baumannii*, carbapenem resistance is conferred by carbapenem-hydrolyzing class D oxacillinases (CHDLs) (8, 15). These include the acquired OXA-23-like, OXA-40-like, OXA-58-like, and OXA-143 oxacillinases, as well as the intrinsic OXA-51-like oxacillinase, of which there are currently 68 variants identified. Although CHDLs exhibit weak carbapenem hydrolysis, they can confer resistance when overexpressed. This is mediated through a combination of naturally low permeability to β-lactams and IS*Aba* elements located upstream of the gene, providing a strong promoter (the OXA-40-like and OXA-143 oxacillinases appear to be exceptions to this) (16, 21).

Molecular typing of isolates obtained from various locations in Europe has shown the existence of three distinct lineages that have been termed European clone I (EUI), EUII, and EUIII (2, 3). More recently, repetitive-sequence-based PCR (rep-PCR) typing using the DiversiLab system has identified eight carbapenem-resistant *A. baumannii* clonal lineages (WW1 to -8) that are distributed worldwide (8). WW1 to -3 have been shown to correspond to EUI to -III (8). A correlation of OXA-69, OXA-66, and OXA-71 to EUI to -III, respectively, has been utilized in a multiplex PCR-based method to identify the three lineages (20). The aim of this study was to investigate the correlation between $bla_{OXA-51-like}$ sequences and worldwide clonal lineages 1 to 8.

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MATERIALS AND METHODS

Bacterial isolates. One hundred two *A. baumannii* isolates were selected from a worldwide collection of imipenem-nonsusceptible *A. baumannii* clinical isolates collected between 2004 and 2010. These had previously been molecularly typed using DiversiLab and assigned to worldwide clonal lineages 1 to 8 or to other (sporadic) genotypes (8). The isolates selected for this study comprised at least eight isolates representing each clonal lineage (Table 1). In addition, 10 isolates with unique DiversiLab genotypes were included. Isolates within a lineage were chosen to represent as many countries of origin as possible.

Sequence group multiplex PCR. Isolates which had been identified as WW1, -2, or -3 using DiversiLab or which were found to be in possession of a bla_{OXA-51} variant that was associated with these lineages were also investigated by multiplex PCR based on amplification of the *ompA*, *csuE*, and $bla_{OXA-51-like}$ genes as previously described (20).

PCR amplification and $bla_{OXA-51-like}$ sequencing. Template DNA was extracted from an overnight culture on blood agar plates. A 1-μl loopful was resuspended in 100 μl sterile water and boiled for 10 min before snap cooling on ice. Amplification of $bla_{OXA-51-like}$ genes was performed as described previously, using primer pair OXA-69A/OXA-69B or preABprom$^+$/OXA-69B when IS*Aba1* was found upstream of the gene (4, 21). PCR products were sequenced in both directions. bla_{OXA-51} variants were identified by BLAST query. To confirm sequences of novel bla_{OXA-51} variants, PCR and sequencing reactions were repeated using Phusion hot-start high-fidelity DNA polymerase (Thermo Fisher Scientific, Schwerte, Germany). Products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced. Novel sequences were assigned by the Lahey β-lactamase database (http://www.lahey.org/Studies/) and submitted to GenBank.

Phylogenetic analysis. Based on the nucleotide sequences covering the whole coding regions of $bla_{OXA-51-like}$ genes, phylogenetic trees were constructed by using the neighbor-joining clustering algorithm and the Jukes-Cantor distance model using Bionumerics 5.1 software (Applied-Maths, St-Martens-Latem, Belgium). An initial analysis looked at clustering of only the bla_{OXA-51} variants sequenced in this study. A second analysis compared clustering of all published bla_{OXA-51} variants.

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TABLE 1 *bla*$_{OXA-51}$ variants and origins of 92 isolates belonging to WW1 to -8

Lineage	*bla*$_{OXA-51}$ variant product (*n*)	Amino acid substitution(s)[a]	Country(ies) of origin
WW1 (EUI)	OXA-69 (7 + 1[b])		Germany, Spain, Pakistan,[b] India, Greece, Italy
	OXA-92 (1)	W234→S	Greece
	OXA-107 (3)[c]	L167→V	Poland
WW2 (EUII)	OXA-66 (13)		UK, Portugal, Australia, Austria, Greece, Ireland, Italy, South Africa, Poland
	OXA-82 (5)[c]	L167→V	USA, Poland, Taiwan
	OXA-172 (1)[c]	I129→V, W222→L	Taiwan
	OXA-201 (1)[c]	L167→V, P130→Q	Spain
	OXA-202 (1)[c]	I129→M	USA
WW3 (EUIII)	OXA-71 (2)		Spain, South Africa
	OXA-113 (7)[c]	L167→V	USA
WW4	OXA-51 (8)		Turkey, Argentina, India, Germany, Brazil, Chile
	OXA-219 (1)[c]	L167→V	Chile
WW5	OXA-65 (12 + 2[b])		Spain,[b] Argentina, USA, Colombia, Venezuela, Germany, Mexico
WW6	OXA-90 (3)		Italy
	OXA-200 (5)[c]	P130→L, W222→L	Honduras
WW7	OXA-64 (10)		Latvia, Switzerland, Venezuela, Mexico, Colombia, Singapore, Germany
WW8	OXA-68 (7)		Spain, Turkey, South Korea, China
	OXA-128 (2)	D68→V	France, Bulgaria

[a] Amino acid changes compared to OXA-69 for WW1, OXA-66 for WW2, OXA-71 for WW3, OXA-51 for WW4, OXA-90 for WW6, and OXA-68 for WW8.
[b] IS*Aba1* upstream of *bla*$_{OXA-51}$ variant in an isolate with an acquired OXA.
[c] IS*Aba1* upstream of *bla*$_{OXA-51}$ variant in an isolate without an acquired OXA.

Nucleotide sequence accession numbers. The nucleotide sequences of the novel *bla*$_{OXA-51}$ variants reported in this paper have been submitted to the EMBL/GenBank database under accession numbers JN790646 (OXA-113b), HQ734811 (OXA-200), HQ734812 (OXA-201), HQ734813 (OXA-202), JN215211 (OXA-219), and JN248564 (OXA-223).

RESULTS

Sequencing of *bla*$_{OXA-51-like}$ genes. To investigate the correlation between *bla*$_{OXA-51}$ variants and DiversiLab clonal lineages, *bla*$_{OXA-51-like}$ genes of 102 clinical isolates were sequenced. Ninety-three isolates were in possession of known *bla*$_{OXA-51}$ variants, and we identified five novel OXA-51-like oxacillinases: OXA-200, OXA-201, OXA-202, OXA-219, and OXA-223. Tables 1 and 2 summarize these results and include the strains' countries of origin. With the exception of *bla*$_{OXA-113}$, which we found to have a nucleotide sequence different from that published (two nucleotide mismatches; henceforth denoted as *bla*$_{OXA-113b}$), DNA polymorphisms were not found in the *bla*$_{OXA-51}$ variants.

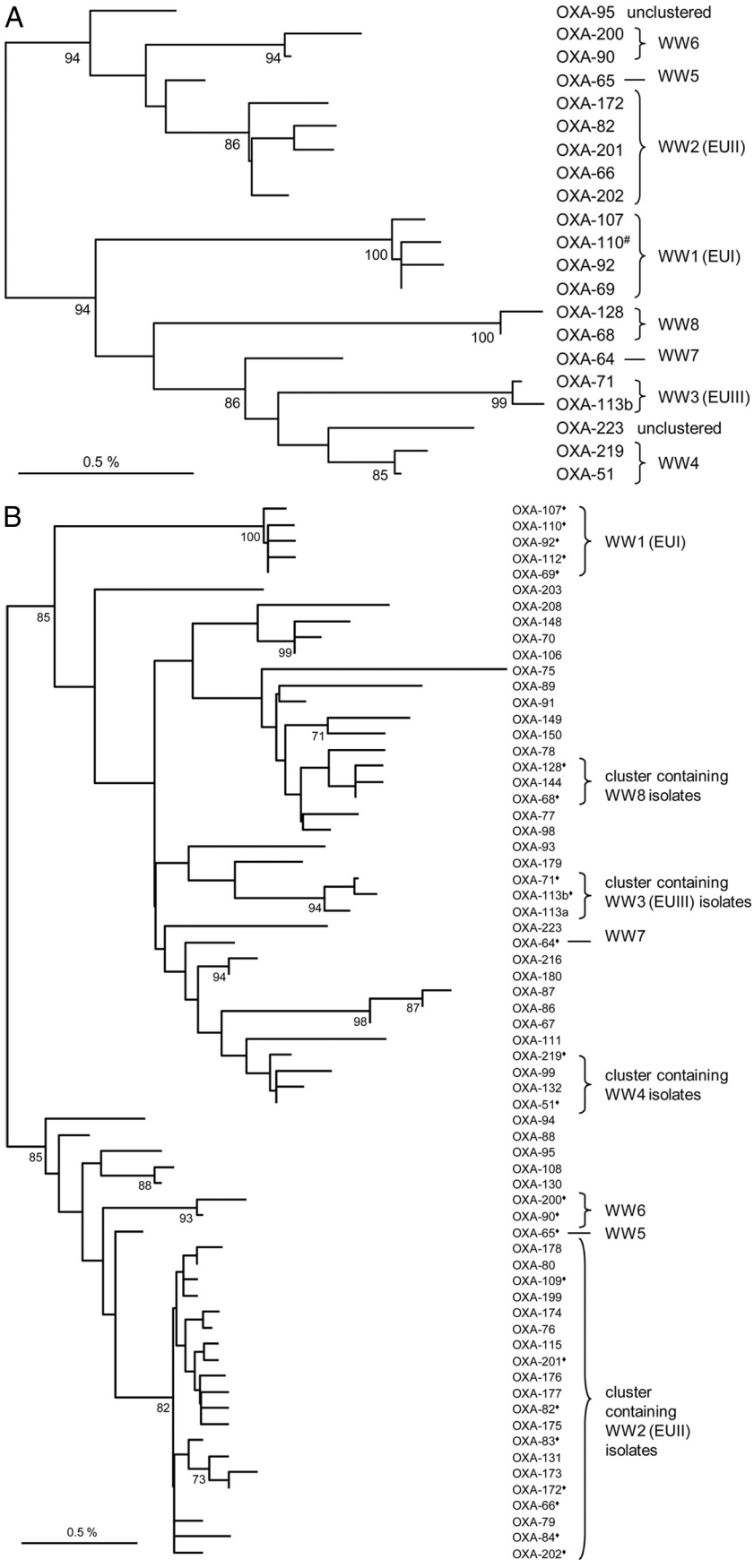
***bla*$_{OXA-51-like}$ typing and DiversiLab clustering.** The *bla*$_{OXA-51}$ variant groups were compared to DiversiLab clusters. Isolates belonging to the same clonal lineage were in possession of similar *bla*$_{OXA-51}$ variants. These had either the same *bla*$_{OXA-51}$ variant or an amino acid variant (Table 1). For example, WW1 isolates were in possession of OXA-69 or variants of this, i.e., OXA-92 (W234→S) or OXA-107 (L167→V) (Table 1).

Interestingly we found that variants of OXA-66, OXA-69, and OXA-71 were identified in carbapenem-resistant isolates without an acquired OXA, and in each case the *bla*$_{OXA-51-like}$ gene was associated with IS*Aba1* (with the exception of *bla*$_{OXA-92}$) (Table 1).

TABLE 2 Isolates that did not cluster in DiversiLab and their expected worldwide lineage (where applicable) based on their *bla*$_{OXA-51-like}$ sequence

bla$_{OXA-51}$ variant (*n*)	Expected worldwide lineage based on *bla*$_{OXA-51}$ variant[a]	Highest similarity (%) to worldwide lineage using DiversiLab	Sequence group	Country(ies) of origin
OXA-69 (2)	WW1	WW1, 87.3	SG2 (EUI, WW1)	Italy, Argentina
OXA-110 (1)	WW1 (see Fig. 1A)	WW1, 87.6	SG2 (EUI, WW1)	Poland
OXA-66 (1)	WW2	WW2, 88.6	SG1 (EUII, WW2)	Australia
OXA-82 (1)	WW2	WW6. 81.3	*bla*$_{OXA51-like}$ band of SG1	Taiwan
OXA-51 (2)	WW4	WW5, 77.9	ND[b]	Chile, Brazil
OXA-68 (1)	WW8	WW8, 84.9	ND	India
OXA-223 (1)	UNC[c]	WW4, 88.8	ND	USA
OXA-95 (1)	UNC	WW6, 79	ND	Singapore

[a] See Table 1.
[b] ND, not determined.
[c] UNC, unclustered.


A
94
94
86
100
94
100
86
99
85
0.5 %
OXA-95 unclustered
OXA-200
OXA-90
WW6
OXA-65
WW5
OXA-172
OXA-82
OXA-201
OXA-66
OXA-202
WW2 (EUII)
OXA-107
OXA-110#
OXA-92
OXA-69
WW1 (EUI)
OXA-128
OXA-68
WW8
OXA-64
WW7
OXA-71
OXA-113b
WW3 (EUIII)
OXA-223 unclustered
OXA-219
OXA-51
WW4
B
100
85
99
71
94
94
98
87
85
88
93
82
73
0.5 %
OXA-107•
OXA-110•
OXA-92•
OXA-112•
OXA-69•
WW1 (EUI)
OXA-203
OXA-208
OXA-148
OXA-70
OXA-106
OXA-75
OXA-89
OXA-91
OXA-149
OXA-150
OXA-78
OXA-128•
OXA-144
OXA-68•
cluster containing WW8 isolates
OXA-77
OXA-98
OXA-93
OXA-179
OXA-71•
OXA-113b•
OXA-113a
cluster containing WW3 (EUIII) isolates
OXA-223
OXA-64•
WW7
OXA-216
OXA-180
OXA-87
OXA-86
OXA-67
OXA-111
OXA-219•
OXA-99
OXA-132
OXA-51•
cluster containing WW4 isolates
OXA-94
OXA-88
OXA-95
OXA-108
OXA-130
OXA-200•
OXA-90•
WW6
OXA-65•
WW5
OXA-178
OXA-80
OXA-109•
OXA-199
OXA-174
OXA-76
OXA-115
OXA-201•
OXA-176
OXA-177
OXA-82•
OXA-175
OXA-83•
OXA-131
OXA-173
OXA-172•
OXA-66•
OXA-79
OXA-84•
OXA-202•
cluster containing WW2 (EUII) isolates

Similarly $bla_{OXA-200}$, a variant of bla_{OXA-90}, and $bla_{OXA-219}$, a variant of bla_{OXA-51}, were also associated with IS*Aba1* and carbapenem resistance. IS*Aba1* was also upstream of one bla_{OXA-69} and two bla_{OXA-65} genes, but the isolates possessing these genes also had an acquired bla_{OXA}.

Ten isolates had unique DiversiLab genotypes (Table 2). Two of these were in possession of bla_{OXA-95} or $bla_{OXA-223}$, which did not cluster with the other bla_{OXA-51} variants (Fig. 1A; Table 2). Five isolates possessed a bla_{OXA-51} variant that suggests that they should cluster with WW1 or WW2. However, DiversiLab did not cluster them with either of these lineages. Therefore, all isolates with OXA-66 or OXA-69 variants were investigated using sequence-based multiplex PCR (Table 2). This confirmed the WW1/WW2 clustering as previously described (8). The four unclustered isolates possessing bla_{OXA-69}, $bla_{OXA-110}$, or bla_{OXA-66} amplified either the sequence group 1 (SG1) or SG2 pattern (Table 2). In addition, the unclustered bla_{OXA-82} isolate was positive for an SG1 $bla_{OXA-51-like}$ gene, while *ompA* and *csuE* were negative.

Phylogenetic analysis. bla_{OXA-51} variant gene sequences could be classified into six distinct clusters and four unique sequences (Fig. 1A). Correlation of OXA-69, OXA-66, and OXA-71 to EUI, -II, and -III, respectively, was shown by the linkage map published by Evans et al., which was based on amino acid sequences (4). OXA-65 was placed in the center of the map, and all variants radiated out from there. However, this linkage map can be misleading. For example, OXA-91 and OXA-95 differ by five amino acids and are distant on the map. OXA-104 and OXA-95 differ by three amino acids and are situated close together on the map. However, at the DNA sequence level, bla_{OXA-91} and bla_{OXA-95} are 98.4% similar, while $bla_{OXA-104}$ is <76% similar to either of these genes. Therefore, we chose to analyze OXA-51-like variants at the DNA level because this may allow for a more sensitive approximation of relatedness.

Figure 1B represents the hypothetical phylogenetic relationship of all bla_{OXA-51} variants published to date, with the exceptions of $bla_{OXA-116}$ and $bla_{OXA-117}$, whose sequences were incomplete, and $bla_{OXA-104}$, which occupied a separate position in the tree, based on very low similarity (<76%) to all other variants (data not shown). Although there was a lack of a clear phylogenetic structure, some putative monophyletic sequence groups were present, as indicated by bootstrapping values of >70% (Fig. 1B). Two large, well-defined clusters encompass bla_{OXA-51} variants associated with WW1 and WW2, respectively.

DISCUSSION

Commonly used methods to identify the clonal relatedness of *A. baumannii* isolates are macrorestriction analysis by pulsed-field gel electrophoresis (PFGE), sequence group typing based on the amplification of three chromosomal genes (20), and multilocus sequence typing (MLST) based on the amplification of seven housekeeping genes (1, 2). Unfortunately these methods are often time-consuming, expensive, or labor-intensive. The major advantage of $bla_{OXA-51-like}$ gene typing is that sequencing is based on a single gene; therefore, this method appears to be an easier, faster, and cheaper way of *A. baumannii* typing. Single-locus sequencing has proved useful with typing of other species, for example, Shiga toxin-producing *Escherichia coli* (STEC) (5) and *Staphylococcus aureus* (*spa* typing) (12, 22). However, some *S. aureus* single-locus methods, such as SCC*mec* and *agr* typing, while useful, are not as discriminatory as *spa* typing (22).

Several studies have also investigated sequence-based $bla_{OXA-51-like}$ gene typing in comparison to other typing methods, where it was shown that $bla_{OXA-51-like}$ gene sequencing corresponded to MLST and sequence group typing (6, 20) but not to PFGE (6). However, the PFGE-derived dendrogram did not fully differentiate between the different EU clonal clusters, highlighting that PFGE is not suited for population studies. In our study, we found a correlation between $bla_{OXA-51-like}$ sequences and worldwide clonal lineages 1 to 8. It was shown that some clonal lineages possess more OXA-51 variants than others, which may result from either the stability of some variants or their association with relatively young lineages. For example, WW5 isolates were geographically widespread but possessed only OXA-65, while WW1 and WW2 were equally widespread but possessed several OXA-51 variants. It was speculated that based on known intraclonal heterogeneity, EUI and -II are relatively old compared to EUIII, and this may explain why they have a greater number of OXA-51 variants (2). Based on this, WW5 is likely to be a more recently established clonal lineage. However, the relative age of a clonal cluster may not be the only factor behind the variability of bla_{OXA-51} variants.

It has been shown that carbapenem resistance is commonly associated with acquired OXAs or overexpression of OXA-51-like oxacillinases (7, 9, 21). We found that the majority of carbapenem-resistant isolates either possessed an acquired OXA or had IS*Aba1* associated with a $bla_{OXA-51-like}$ gene. Interestingly, IS*Aba1* was associated predominantly with $bla_{OXA-51-like}$ genes which encoded amino acid variants of OXA-66, OXA-69, OXA-51, OXA-71, and OXA-90 but mainly where it was the only carbapenem resistance mechanism detected. Not only do insertion sequence (IS) elements lead to overexpression, but published data suggest a role of IS elements in the evolution of β-lactamases. For example, an *in vivo* mutation was described in *A. baumannii* that converted the acquired $bla_{OXA-164}$ into bla_{OXA-58} and was associated with carbapenem therapy and the presence of IS*Aba3* (10). Similarly, it was shown in *E. coli* that the IS*Ecp1*-associated extended-spectrum β-lactamase CTX-M-3 exhibited an amino acid substitution after selection on ceftazidime (18). It can therefore be hypothesized that carbapenem therapy may play a role in the selection of bla_{OXA-51} variants when it is the sole carbapenem resistance determinant and associated with IS*Aba1*-mediated overexpression.

As the association between DiversiLab types and bla_{OXA-51} variants suggests the coevolution of $bla_{OXA-51-like}$ sequences with other parts of the *A. baumannii* genome (seen as different rep-PCR patterns), it would be interesting to further investigate isolates carrying each of the known OXA-51 variants to gain further insights into the correlation between sequence-based $bla_{OXA-51-like}$

FIG 1 (A) Unrooted neighbor-joining tree based on $bla_{OXA-51-like}$ nucleotide sequences of 102 clinical isolates. Bootstrap percentages of >70% after 1,000 replicates are shown. Horizontal bar, 0.5% sequence divergence. #, the isolate possessing OXA-110 did not cluster with WW1 to -8 by rep-PCR but was positive for SG2 (WW1). (B) Unrooted neighbor-joining tree based on nucleotide sequences of all published bla_{OXA-51} variants except $bla_{OXA-116}$, $bla_{OXA-117}$, and $bla_{OXA-104}$. Horizontal bar, 0.5% sequence divergence. Bootstrap percentages of >70% after 1,000 replicates are shown. ♦, bla_{OXA-51} variants ($bla_{OXA-109}$, $bla_{OXA-112}$, bla_{OXA-83}, and bla_{OXA-84}) that have previously been shown to correlate with EUI to -III (6, 20).

typing and other typing methods. The results for our rep-PCR-unclustered isolates indicate that amplification of repetitive regions of the genome may not always be in agreement with sequence-based multiplex PCR. Repetitive sequences may be subject to rapid changes brought about by recombination events, which may explain the lack of correlation between the two methods with these strains. In a recent study, recombination hot spots were found to include genomic regions that encode proteins associated with cell surface molecules but to our knowledge were not associated with $bla_{\text{OXA-51-like}}$ genes (17). Therefore, the use of alternative methods such as the Bartual and/or Pasteur MLST schemes may help to resolve this issue (1, 2).

In summary, despite the variation in DNA sequences, we observed a striking correlation between $bla_{\text{OXA-51}}$ monophyletic groups and *A. baumannii* worldwide clonal lineages 1 to 8. Therefore, sequencing of $bla_{\text{OXA-51-like}}$ genes has the potential to contribute to the population analysis of *A. baumannii* and be used to identify not only European clones I to III but also *A. baumannii* isolates belonging to worldwide clonal lineages 1 to 8.

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